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The Antitumor Effects of Selenium Compound $Na_5SeV_5O_{18}\cdot 3H_2O$ in K562 Cell

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With an approach to study the anti-tumor effects and mechanism of selenium compound, we investigated the anti-tumor activity and mechanism of $Na_5SeV_5O_{18}\cdot 3H_2O$ (NaSeVO) in K562 cells. The results showed that 0.625~20 mg/L NaSeVO could significantly inhibit the proliferation of K562 cells *in vitro* in a time- and concentration-dependent manner as determined by microculture tetrazolium (MTT) assay, the IC50 values were 14.41 (4.45-46.60) and 3.45 (2.29-5.22) mg/L after 48 h and 72 h treatment with NaSeVO respectively. *In vivo* experiments demonstrated that i.p. administration of 5, 10 mg/kg NaSeVO exhibited an significant inhibitory effect on the growth of transplantation tumor sarcoma 180 (S180) and hepatoma 22 (H22) in mice, with inhibition rate 26.8% and 58.4% on S180 and 31.3% and 47.4% on H22, respectively. Cell cycle studies indicated that the proportion of G0/G1 phase was increased at 2.5 mg/L while decreased at 10 mg/L after treatment for 24, 48 h. Whereas S phase was decreased at 2.5-5 mg/L and markedly increased at 10 mg/L after treatment for 48 h. After treatment for 24 h, 10 mg/L NaSeVO also markedly increased S and G2/M phases. Take together, the result clearly showed that NaSeVO markedly increased S and G2/M phases at 10 mg/L. The study of immunocytochemistry showed that the expression bcl-2 is significantly inhibited by 10 mg/L NaSeVO, and bax increased. Morphology observation also revealed typical apoptotic features. NaSeVO also significantly caused the accumulation of Ca^{2+} and Mg^{2+}, reactive oxygen species (ROS) and the reduction of pH value and mitochondrial membrane potential in K562 cells as compared with control by confocal laser scanning microscope. These results suggest that NaSeVO has anti-tumor effects and its mechanism is attributed partially to apoptosis induced by the elevation of intracellular Ca^{2+}, $Mg^{2+}$and ROS concentration, and a reduction of pH value and mitochondria membrane potential (MMP).

Key words: Selenium compound, Anti-tumor action, Apoptosis

INTRODUCTION

As a trace element, selenium is essential for nutrition and exhibits a wide range of biological functions. It was also shown to have anticarcinogenic or preventive chemicals from inducing tumors, as reported by Shamberger (1985) and Rojas *et al.* (1999). Epidemiological studies have shown that populations with a low selenium intake and low plasma selenium levels have an increased incidence of cancer, including cancer of the breast, lung, stomach, bladder, ovaries, pancreas, thyroid, esophagus, head and neck, cerebellum and melanoma, etc. (Huang *et al.*, 1999; Burney *et al.*, 1997; Glattre *et al.*, 1989; Jaskiewicz *et al.*, 1988; Westin *et al.*, 1989; Philipov and Tzatchev, 1988; Sinha and El-Bayoumy, 2004). Therefore, people have focused their attention on searching for potent selenium compounds, which possess higher anti-tumor efficacy and lower tissue toxicity. Natural and synthetic selenium compounds have been examined as chemopreventive agents in several animal tumor models (Ip, 1998; El-Bayoumy, 2001). Several studies performed *in vitro* suggest that induction of apoptosis and/or inhibition of cell growth can account for cancer prevention by selenium compounds (Sinha *et al.*, 1993; Gasparian *et al.*, 2002; Ip *et al.*, 2002).

Apoptosis, also known as programmed cell death, plays a fundamental role in the development of multicellular organisms and numerous physiological processes. Some

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researchers (Lowe and Lin, 2000; Wang, 1999) reported that genetic mutations culminating in the disturbance of apoptosis or derangement of apoptosis-signaling pathways seem to be an essential factor of carcinogenesis. Kornblau (1998) regarded that the induction of apoptosis of cancer cells is one of the most important methods for cancer treatment, and many anti-cancer agents have been reported to induce apoptosis of cancer cells. So induction of apoptosis and inhibition of cell proliferation are considered as important cellular events that can account for the cancer preventive effects of selenium (Kamesaki, 1998). One of the mechanisms of cytotoxicity caused by chemotherapeutic agents is *via* free radical dependent mechanisms (Benner *et al.*, 1997), and apoptosis induced by changes in intracellular ion concentrations such as Ca^{2+}, Mg^{2+}, and H^+ is another mechanism of cytotoxicity (McConkey and Orrenius, 1996; Cotter and Fernanded, 1993; Simon *et al.*, 1994). The aim of this study was to test whether NaSeVO, a novel synthetic selenium compound, has anti-tumor activity in K562 cells, and to explore its mechanism of actions.

MATERIALS AND METHODS

Drugs and chemicals

$Na_5SeV_5O_{18}\cdot 3H_2O$ (NaSeVO), orange yellow crystal, was kindly provided by Laboratory of Organic Chemistry, Lanzhou University (Lanzhou, China). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). RPMI-1640 medium was obtained from GIBCO BRL (Grand Island, NY, U.S.A.). Bovine serum was purchased from Hangzhou Sijiqing Biotechnology Co. (Hangzhou, China). Monoclonal antibody Bcl-2, 2D2 and sabc kit were purchased from Zymed labortrory. Fluo-3/AM, Mag-Fluo-4/AM, Carboxy SNARF-1/AM, 2', 7' -dichlorofluorescin diacetete and Mito Tracker Green FM were purchased from Molecular Probes Co. (Eugene, Oregon, U.S.A.). Other chemicals were analytical purity.

Cell culture and MTT assay *in vitro*

K562 cell line was purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in complete RPMI-1640 medium containing 10% heat-inactivated bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2, and routinely passaged every other day.

Cytotoxicity was measured by microculture tetrazolium (MTT) assay (Mosmann, 1983). Briefly, exponentially growing cells were washed and resuspended in complete RPMI-1640 medium to a concentration of 1×10^8 cells/L, and 100 μL aliquots of cells containing NaSeVO 0.625-20 mg/L were seeded in quadruplicate into a 96-well flat bottom microculture plate (Costar, Corning, U.S.A.) for the varying periods of time. At the end of the incubation period, MTT (5 g/L, w/v, in PBS pH 7.4) 10 μL was added to each well and further cultured for the last 4 h, then SDS 100 μL (10%, w/v, in 0.01 M HCl) was added and mixed thoroughly to dissolve formazan crystals at 37°C. Optical density was read on a Microplate Reader (Elx800, Bio-TEK instruments, Inc, U.S.A.) at 570 nm after shaking plates for 5 min.

Cell cycle analysis by flow cytometry

In order to detect cell cycle distribution of K562 cell induced by NaSeVO, the percentage of cell cycle in K562 cells was analyzed by flow cytometry. After treatment with NaSeVO 0.625-20 mg/L for designed time, cells were washed in PBS and fixed in ice-cold 70% ethanol at 4°C for at least 24 h. The cells were washed in PBS (pH 7.4), and stained with propidium iodide (PI) solution containing PI 50 mg/L and RNase 50 mg/L in PBS at room temperature in the dark for 30 min. The samples were read on a Coulter Epics XL flow cytometry (Beckman-Coulter Inc, Fullerton, CA, U.S.A.). The percentage of cell cycle in G_0/G_1, S and G_2/M phase was calculated using Multicycle software (Phoenix Flow System, San Diego, CA, U.S.A.).

Animals and tumor transplantation *in vivo*

Female Kunming mice weighing 18.0-22.0 g were purchased from the Experimental Animal Center of Lanzhou University and provided with diet of pellets and water *ad libitum*. All experiments involving mice were approved by the Institutional Animal Care and Use Committee. According to protocols of mouse tumor xenograft models (Wand, 1997), Kunming mice were inoculated subcutaneously (s.c.) into right axillary fossa on day 0 with 3×10^6 viable sarcoma 180 (S180) or hepatoma 22 (H22) cells each mouse in a volume of 0.2 mL. On day 1 mice were randomly divided into four experimental groups (n=10 mice for each group): Control and NaSeVO groups, and were treated intraperitoneally (i.p.) with 0.9% NaCl, and NaSeVO 1, 5,10 mg/kg daily, respectively. The tumor-bearing mice implanted with S180 or H22 were sacrificed 12 days after inoculation and the tumors were removed and immediately weighed.

Immunohistochemistry (IHC) assay

Immunohistochemistry assay was used to detect the expression of Bax and Bcl-2. After treatment with NaSeVO 10 mg/L for 24 h, cells were washed in PBS three times, smeared, air drying and fixed, then operated according to the manufacturer's instructions and examined with microscope.

Morphological features of apoptosis

Inverted microscope: K562 cells (1×10^6 cells/mL) were grown in medium containing 0.625-20 mg/L NaSeVO for 24 h, then stained with right-Giemsa and observed under inverted microscope.

Electron microscope: K562 cells treated with NaSeVO 10 mg/L for 24 h were prefixed in cacodylate-buffered glutaraldehyde (2%), post-fixed in 1% osmium tetraoxide, dehydrated in graded series of alcohol, and embedded in Epon (PolyBed 812). Sections were stained with uranyl acetate and lead citrate and were examined with EM-1230 electron microscope (Japan).

Determination of intracellular Ca^{2+}, Mg^{2+}, ROS concentration, pH value and MMP

Following treatment as described above, K562 cells were washed twice in ice-cold PBS, and then loaded with Fluo-3/AM (5 μmol/L), Mag-fluo-4 (5 mmol/L), Carboxy SNARF-1/AM (10 μmol/L), 2', 7'-dichlorofluorescin diacetete and Mito Tracker Green FM (1.25 μmol/L), respectively, for 45 min at 37°C according to the manufacturer's instructions. The cells were again washed twice in PBS. The fluorescence intensity changes of intracellular Ca^{2+}, Mg^{2+}, ROS, pH value and MMP were measured using confocal laser scanning microscopy.

Statistical analysis

The two-tailed Student's t-test was employed to assess the significance of the data. The data are presented as mean± SD. The level of significance was taken at $p<0.05$.

RESULTS

Anti-proliferation activity of NaSeVO *in vitro*

As shown in Table I, NaSeVO 0.625~20 mg/L significantly inhibited proliferation of K562 cells *in vitro* in a time and dose-dependent manner, The IC_{50} values were 14.41 (4.45-46.60) mg/L and 3.45 (2.29-5.22) mg/L after treated with NaSeVO for 48 h and 72 h, respectively.

Anti-tumor activity *in vivo*

The anti-tumor effect of NaSeVO *in vivo* was evaluated by the inhibition rate of tumor mass. Data (Table II) showed that i.p. NaSeVO 5 and 10 mg/kg had a significant anti-tumor effect on the growth of S180 with inhibition rate 26.8% and 58.4%, and 31.3% and 47.4% on H22, respectively.

Effect of NaSeVO on cell cycle distribution of K562

Fig. 1 showed the proportion of G_0/G_1 phase was decreased, whereas G2/M and S phase were increased after treatment for 24, 48 h.

Eexpression of Bax and Bcl-2

Buffy precipitate appearanced in endochylema or cell membrane was regarded as positive expression. The study of immunocytochemistry shown that the expression of bcl-2 is significantly inhibited by NaSeVO 10 mg/L, and bax increased (Fig. 2).

Morphological features of apoptosis

Typical apoptosis character was present in the K562 cells treated with NaSeVO for 24 h. Nuclear condensation, chromosome fragmentation and apoptosis bodies were observed by inverted microscope. The electron microscopic observation also revealed typical apoptotic features, including shrinkage of cellular and nuclear membranes, condensed heterochromatin around the nuclear periphery, and cytoplasmic vacuolation in the K562 cells treated with NaSeVO 10 mg/L for 24 h (Fig. 3).

Table I. *In vitro* anti-tumor effects of NaSeVO on K562 cells after 24, 48 and 72 h treatment

Concentration /mg·L^{-1}	24 h *OD_{570}	48 h *OD_{570}	IC_{50} /mg·L^{-1}	72 h *OD_{570}	IC_{50} /mg·L^{-1}
0	0.413±0.018	0.682±0.033	14.41 (4.45- 46.60)	1.070±0.107	3.45 (2.29-5.22)
0.625	0.347±0.011**	0.516±0.016**		0.813±0.033**	
1.25	0.332±0.004**	0.514±0.024**		0.763±0.064**	
2.5	0.331±0.006**	0.489±0.009**		0.680±0.060**	
5	0.330±0.015**	0.488±0.011**		0.503±0.011**	
10	0.302±0.014**	0.424±0.014**		0.334±0.012**	
20	0.281±0.006**	0.267±0.029**		0.065±0.009**	

Note: MTT assay was used to measure the cytotoxic effect of NaSeVO in K562 cells. Results were expressed as the mean ± SD of three experiments. * $p<0.01$ *vs*. control. The potency of drug was determined by the IC_{50} values (50% growth-inhibition concentration).
*OD, optical density.

Table II. Inhibitory effects of $Na_5SeV_5O_{18}\cdot3H_2O$ on the growth of S180 or H22 in tumor-bearing mice

Dose /mg·kg^{-1}	S180		H22	
	Tumor Weight /g	IR /%	Tumor Weight /g	IR /%
0	1.762±0.642	-	1.113±0.447	-
1	1.459±0.393	17.2	1.104±0.302	8
5	1.290±0.214*	26.8	0.765±0.254*	31.3
10	0.733±0.390**	58.4	0.585±0.132*	47.4

Note: On day 0 female Kunming mice were inoculated s.c.with 3×10^6 viable S180 or H22 tumor cells each mouse. On day 1 they were treated i.p.with NaSeVO 1, 5, 10 mg/kg daily, respectively. The mice were sacrificed 12 days after inoculation and the tumors were removed and weighed. Inhibition rate (IR %) = (1 - Tumor weight $_{Treated}$ / Tumor weight $_{Control}$) × 100%. Results are expressed as mean ± SD of 10 mice. * $p<0.05$, ** $p<0.01$ *vs*. control.

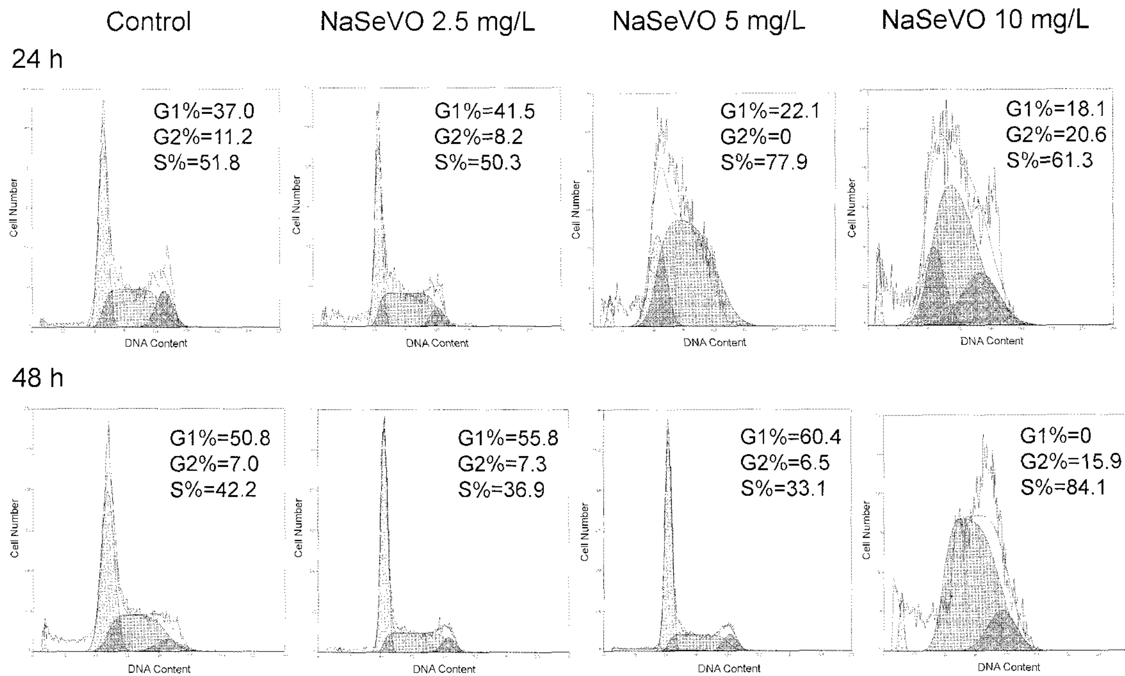

Fig. 1. Cell cycle analysis in treated K562 cells by flow cytometry

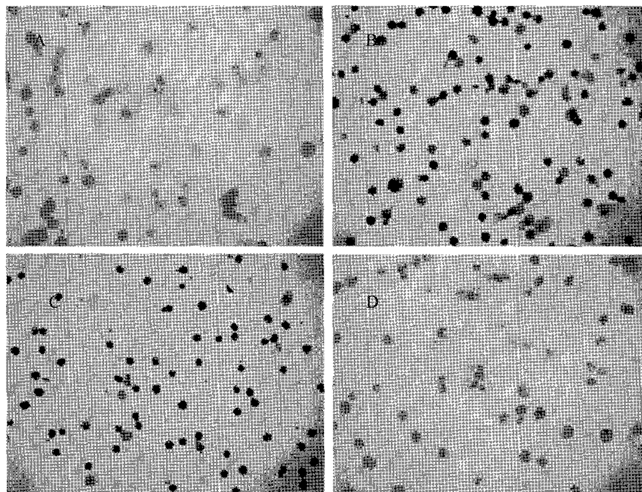

Fig. 2. Expression of Bax and Bcl-2 was observed by Immunohistochemistry (IHC) assay. Buffy precipitate appearanced in endochylema or cell membrane was regarded as positive expression. A and B: Expression of Bcl-2, A: untreated cells, B: treated cells; C and D: Expression of Bax, C: untreated cells, D: treated cells.

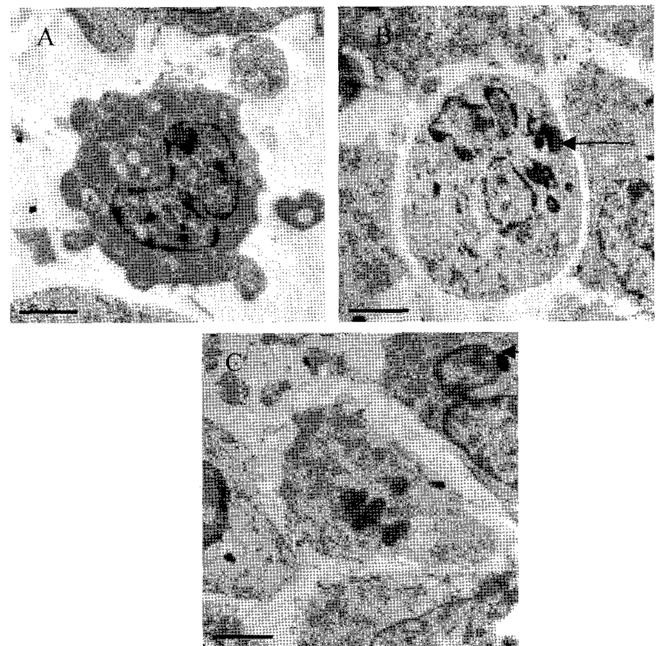

Fig. 3. Morphological observation of K562 cells by electron microscopy treated with NaSeVO 5, 10 mg/L for 24 h. A: untreated cells (8000×); B and C: treated with NaSeVO 5, 10 mg/L (8000×), respectively. Arrowhead indicated nuclear body. Scale bar =1 μm.

Effect of NaSeVO on intracellular Ca^{2+}, Mg^{2+} and ROS concentration, pH value and MMP

As shown in Fig. 4 and Table III, the fluorescence intensity of intracellular Ca^{2+} and Mg^{2+} was greatly increased after treatment with NaSeVO as compared with control group. Similar to the change of intracellular Ca^{2+} and Mg^{2+}, fluorescence intensity of intracellular ROS also increased. However, treatment with NaSeVO markedly lowered the fluorescence intensity of intracellular pH value and mitochondrial membrane potential.

DISCUSSION

Our results clearly demonstrated that NaSeVO signifi-

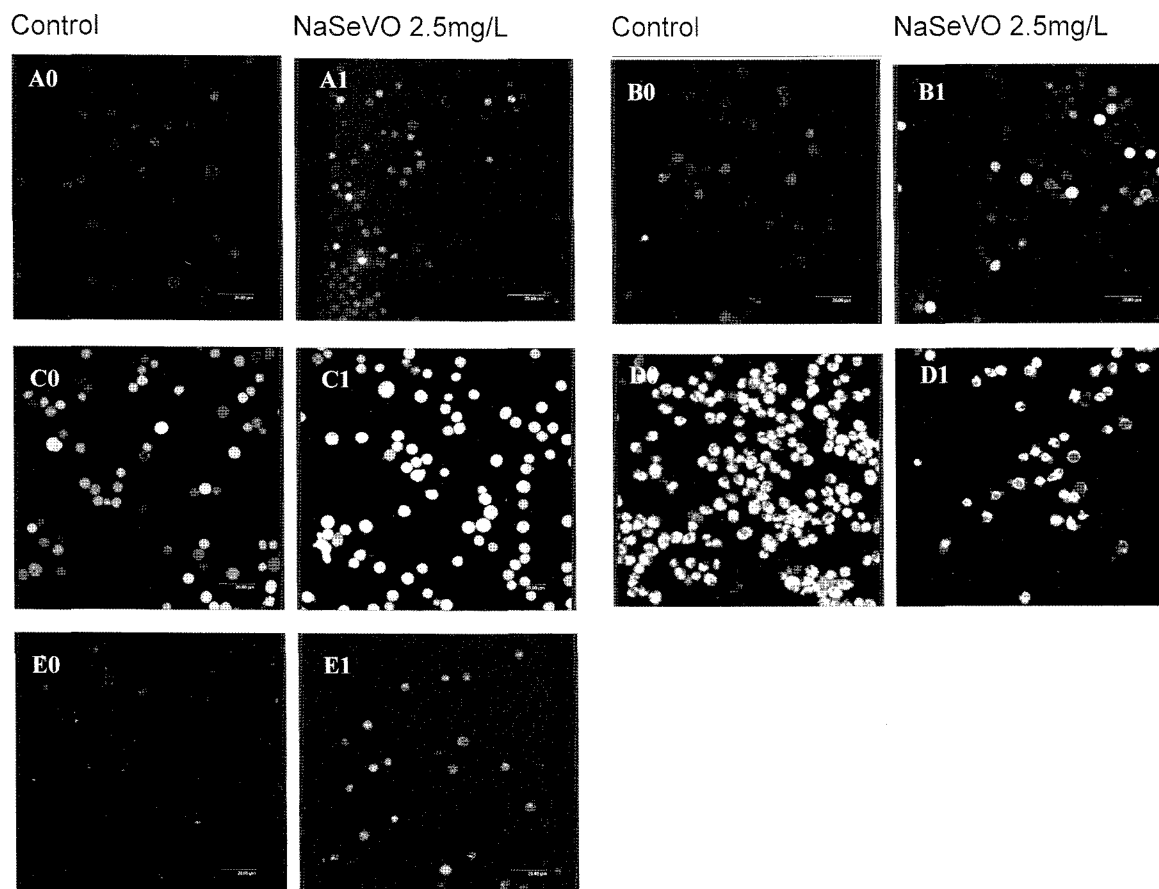

Fig. 4. Fluorescent intensity of intracellular Ca^{2+}, H^{+}, ROS, MMP and Mg^{2+} in K562 cells by confocal laser scanning microscopy after 24 h treatment with NaSeVO. 0: control; 1: treatment with NaSeVO 2.5 mg/L. A, B, C, D and E: fluorescent intensity of intracellular Ca^{2+}, H^{+}, ROS, MMP and Mg^{2+}, respectively. Scale bar =20 μm.

Table III. Fluorescence intensity of intracellular ion concentration and MMP in K562 cells

Concentration / $mg \cdot L^{-1}$	Intracellular fluorescent intensity				
	Ca^{2+}	Mg^{2+}	ROS^{-}	H^{+}	MMP
0	25.25±6.78	45.96±6.98	126.71±7.74	70.28±4.02	220.14±3.88
1.25	33.42±1.42	81.25±6.59**	179.75±3.98**	87.05±7.15*	219.09±8.44
2.5	41.84±6.16*	105.30±8.68**	199.82±9.96**	132.11±9.83*	209.19±5.94
5	57.98±6.70**	158.43±9.57**	212.61±7.26**	148.43±8.29**	198.07±3.27**
10	67.51±6.19**	199.45±9.07**	221.52±9.03**	214.19±4.00**	152.18±4.56**

Note: After 24 h treatment, K562 cells were loaded with fluorescence probe for 45 min at 37°C. The fluorescence intensity of intracellular Ca^{2+}, Mg^{2+}, ROS, H^{+}, and MMP in K562 cells were determined by confocal laser scanning microscopy. Results are expressed as the mean ± SD of three experiments. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* control.

cantly inhibited proliferation of K562 cells *in vitro* and S180 and H22 *in vivo* in a concentration-dependent manner. Cell cycle analysis showed NaSeVO induced K562 cells accumulated in S and G_2/M phase, as reported by Sinha and El-Bayoumy, (2004) that several selenium compounds could arrest cells in either phase. NaSeVO also induced typical apoptotic features. It is known that apoptosis is important cellular events that can account for the cancer preventive effects of selenium (Kamesaki, 1998).

Apoptosis is regarded as an active suicidal response to various physiological or pathological stimuli including anti-cancer agents. It is not only a genetically controlled mechanism essential for development, maintenance of tissue homeostasis and elimination of unwanted or damaged cells such as tumor cells (Sinha and El-Bayoumy, 2004), but also a commonly accepted mechanism of anti-tumor

effect of chemotherapeutic drugs. In addition, mitochondria disruption play a major role during apoptosis induction, resulting in membrane permeability transition and the release of mitochondrial apoptogenic factors such as mitochondrial cytochrome c, which is released into the cytosol in response to the apoptotic signals (Nicholls and Ward, 2000). So the perturbations in mitochondrial membrane potential could be one of the most conspicuous manifestations of apoptosis. Evaluation of the mitochondrial function during induction of apoptosis was recorded through changes in its decreased MMP. Monitoring the MMP by using fluorescent probe has generally been adopted as an indicator of cell apoptosis (Nicholls and Ward, 2000; Bolduc *et al.*, 2004). Our results showed that NaSeVO markedly induced the collapse of intracellular MMP. This was in agreement with the report (Shilo et al., 2003) that showed selenite induced mitochondrial permeability transition and provoked the release of cytochrome c. This implies that the decreased MMP may contribute to apoptosis induced by NaSeVO.

Many of the pro-apoptotic and anti-apoptotic members of the Bcl-2 family, such as bax and bcl-2 regulated apoptosis though the mitochondria, either by interacting with each other, or through direct interactions with the mitochondrial membrane. So the expression of Bcl-2 and bax was used as symbolized gene of apoptosis. The immunocytochemistry shows that the expression of bcl-2 is significantly inhibited by NaSeVO 10 mg/L, and bax increased.

An excellent approach to apoptosis research has focused on changes in intracellular ion concentrations. It is presumed that perturbations in intracellular ion homeostasis could be the other conspicuous manifestation of apoptosis. Because of the multitude of proteins which are activated in the apoptotic cascade, and which invariably depend on the existence of certain intracellular ions. As reported by Munaron *et al.* (2004) that cell proliferation and differentiation have been linked to the stimulation of the intracellular Ca^{2+} signal. Perturbation of intracellular Ca^{2+} appears to be a common mechanism of apoptosis. Particular emphasis has been placed on the influence of Ca^{2+} and Mg^{2+} ions. Ca^{2+} is one of the most important intracellular messengers in modulating cell growth and differentiation, and plays an essential role in the induction of apoptosis. The role of Ca^{2+} as an intracellular messenger is incomplete without the coexistence of internal Mg^{2+} ions. The regulatory function of Ca^{2+} is carried out in synergy with the structural function of Mg^{2+}. Consistent to this implication, in this study, it was showed that NaSeVO markedly increased intracellular Ca^{2+}, Mg^{2+} concentration. These results suggest that Mg^{2+} may be adjunct to Ca^{2+} ions responsible for apoptosis induction. These results were consistent to the other researches (Wang *et al.*, 2002; Zhong and Oberley, 2001).

The changes of intracellular ion homeostasis such as Ca^{2+} and Mg^{2+} accumulation may induce mitochondrial apoptosis and lower its membrane potential. Ca^{2+}, Mg^{2+} play a crucial role in governing the morphological and biochemical changes attributed to apoptotic cell death (Cain *et al.*, 1994). So the perturbations in intracellular ion homeostasis, pH value and MMP could be a conspicuous manifestation of apoptosis.

Apoptosis are closely associated with Ca^{2+}, Mg^{2+} and MMP. Another crucial factor, ROS has been implicated as a main mediator of apoptosis in many different cellular systems (Jacobson, 1996). ROS may induce cell death by themselves or act as intracellular messengers during the cell death induced by various other kinds of stimuli (Jung *et al.*, 2001). Some researchers (Foster and Sumar, 1997) regarded that excess of selenium was likely to create an over oxidized environment in cells and cause cell dysfunction and apoptosis. Our experiment showed intracellular ROS was significantly increased by treatment with NaSeVO, it can be suggested that apoptosis induced by NaSeVO is closely related to the increase of intracellular ROS level and possible affects intracellular redox status.

In summary, the present study demonstrated that NaSeVO significantly inhibited the growth of K562 cells *in vitro* and S180 or H22 *in vivo,* and induced K562 cells accumulated some certain phase at different concentration. The antitumor mechanism may relate to apoptosis induced by accumulation of intracellular Ca^{2+}, Mg^{2+} and ROS concentration, and reducing pH value, mitochondrial membrane potential.

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